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A Stability-Indicating HPLC-PDA Method for Simultaneous Determination of Paracetamol, Caffeine, Ibuprofen and their Degradation Products in Solid Dosage Forms

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ABSTRACT

Purpose of this study was to develop a stability-indicating RP-HPLC method for routine analysis of Paracetamol (PARA), Caffeine (CAF) and Ibuprofen (IBU) in their combined solid dosage forms. The new RP-HPLC method was validated as per ICH, FDA and USP guidelines with respect to accuracy, precision, specificity, linearity, solution stability, robustness, sensitivity and system suitability. The method was developed by using a binary gradient mode of phosphate buffer (pH 7.2) and acetonitrile at a flow rate of 1.3mL/min for 15 minutes over C-18 (ODS, 150 x 4.6 mm, 5µm) column at ambient temperature. Injection volume was 20µL for both standard and sample solutions and the eluents were monitored with UV detection at 230nm.Accuracy was determined by the recovery tests of the drugs and found to be within a range of 99.89% to 100.33%. Intraday and inter-day precisions were demonstrated by a relative standard deviation being far less than maximum allowable limit (2.0%, according to FDA). The method showed linear response with a correlation coefficient (r^2) value of 0.999 for all three drugs. Forced degradation studies in acidic, basic, oxidation and reduction media were carried out to establish the stability indicating tolerance of this method. Specificity was shown by the separation of drugs with high degree of resolution between them and absence of any interference from the excipients or degradation products. This method was successfully applied to assay the drugs in tablets and capsules. Hence this newly developed method can be considered suitable and reliable for the routine analysis of PARA, CAF and IBU in their solid dosage forms.

Keywords: Paracetamol**,** Caffeine**,** Ibuprofen**,** RP-HPLC**,** Simultaneous quantification.

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INTRODUCTION

Ibuprofen (IBU) is a non-steroidal anti-inflammatory drug (NSAID). It is cyclooxygenage inhibitor and is used for symptomatic treatment of rheumatoid arthritis, osteoarthritis, alkylosing spondylitis, acute gouty arthritis, and as an analgesic for acute tendinitis, bursitis and primary dysmenorrhoea[1]. IBU is known to have an anti platelet effect, though it is relatively mild and short-lived when compared with that of aspirin or other better-known antiplatelet drugs. Paracetamol or acetaminophen(PARA) is a widely used over-the-counter analgesic (pain reliever) and antipyretic (fever reducer). It is an effective alternative to aspirin as an analgesic-antipyretic agent[1]. PARA is used to treat many diseases such as headaches, muscle aches, arthritis, backache, toothaches, colds and fevers. Caffeine (CAF), the most common stimulant, is used in this drug combination to speed up the action of the drug as a synergist to PARA[2, 3]. IBU belongs to arylpropionic acid derivatives and is chemically (*RS*)-2-(4-(2-methylpropyl) phenyl) propanoic acid. PARA belongs to para aminophenol derivatives and is chemically *N*-(4-hydroxyphenyl) acetamide. CAF is chemically 1,3,7-Trimethyl-1*H*-purine-2,6(3*H*,7*H*)-dione 3,7-Dihydro-1,3,7-trimethyl-1*H*-purine-2,6-dione (Figure 1).

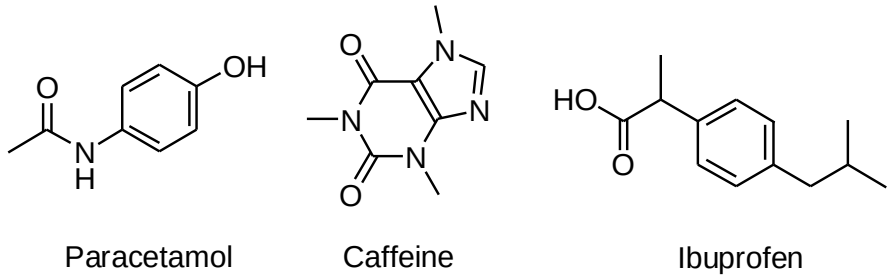

Figure 1: Basic chemical structure of the three drugs

These three drugs are nowadays used in combination in various strengths as tablets and capsules. So development of an analytical method for assay of these drugs in pharmaceutical dosage form is of utmost necessity to confirm the quality of tablets or capsule with respect to assay, content uniformity and dissolution. There are methods for assaying these drugs singly in single dosage form described in United States Pharmacopoeia (USP) and British Pharmacopoeia (BP). Various analytical methods have also been stated for combination dosage form of PARA and CAF[4-8] and IBU and PARA[9-14]. An exhaust search was made in order to find out simultaneous determination of these three drugs in their combined formulation and found only one spectrophotometric - chemometric method[15]. To the best of our knowledge, there is no single HPLC method for simultaneous determination of PARA, CAF and IBU in combined dosage form so far. Therefore, we put our effort to develop a simple and simultaneous and stability-indicating HPLC analytical

method for the routine analysis as well as research work of these drugs combinations. The developed method was validated maintaining the guidelines of Unite States Pharmacopoeia (USP), Food and Drug Administration (FDA) and International Conference on Harmonization (ICH).

MATERIALS AND METHOD

Working standards of the drugs were kind gift of Aristopharma Limited, Bangladesh. HPLC grade acetonitrile was obtained from Active Fine Chemicals Ltd., Bangladesh.

HPLC System and Chromatographic Conditions

High Performance Liquid Chromatographic system (Shimadzu-UFLC Prominence), equipped with an auto sampler (Model- SIL 20AC HT) and PDA detector (Model-SPD 20A) was used for the analysis. The data was recorded using LC-solutions software. Analytical reversed phase C-18 column [4.6 x 150 mm, 5µm] was used to analyze the standards and samples. All analyses were done at ambient temperature under binary gradient condition. The mobile phase was run at a flow rate of 1.3 mL/min for 15 minutes. The injection volume was 20 µL for standard and samples. Before analysis, every standard and sample was filtered through 0.22 µm filter tips. The column eluent was monitored with UV detection at 230nm.

Preparation of Buffer Solution of Mobile Phase (pH 7.2)

1.3g of di-potassium hydrogen phosphate was dissolved in 500mL of nano-pure water and this was marked as Solution-A. Then 1.0 g of potassium di-hydrogen phosphate was dissolved in 300mL nano-pure water and was marked as Solution-B. Finally solution-B was added carefully in to solution-A until the pH reached 7.2 (±0.1).This solution was filtered through a nylon membrane filter (0.20 µm) using a Millipore glass filter holder. Thus the buffer solution of mobile phase was obtained.

Preparation of Standard Mixture

A blend of buffer mobile phase and acetonitrile in a ratio of 65:35was used as a solvent. A stock standard solution was prepared by dissolving 50mg of PARA, 20mg of CAF and 40mg IBU in to a100mL.Then 5mL of this solution was taken into another 50mL volumetric flask and diluted with the solvent to get nominal working standard solution of 50µg/mL PARA, 40 µg/mL IBU and 20µg/mL CAF. The stock solution was further diluted with the solvent to get another six working standard solutions having a concentration ranging from 50% to 200% of the nominal concentration. All the solutions were filtered through syringe filter (0.22µm) in to vials.

Preparation of Sample Solution

For quantification of the drugs in tablets, 20 tablets were crashed and grinded to make fine powder.

Then desired amount of powdered tablet was taken into volumetric flask, diluted with the solvent and sonicated for10 minutes to dissolve the particles completely. Solutions were diluted to the suitable concentration for analysis. In case of capsule, contents of 20 capsules were taken together and mixed properly. Then desired amount of powder was taken in to volumetric flask and diluted with solvent. All the solutions were filtered through 0.22µmsyringe filter into vial.

Method Validation

The method validation was made according to guidelines about the validation and verification of analytical methods as described in the United States Pharmacopoeia, Food and Drug Administration (FDA) and International Conference on Harmonization (ICH)[16-18]. The specificity of the LC method was evaluated to ensure that there was no interference from the degradation products, excipients or other impurities in the pharmaceutical formulation. Stability of drug combination in diluting solvent and mobile phase was checked by rendering the test solutions in tightly capped vials at room temperature and in refrigerator (at 5ºC) for 48hours. The solutions were analyzed by the developed method at 0 hour, 24 hour and48hour. Different concentrations of working standard solution (from 50% to 200% of nominal concentration) were prepared from stock solution. The average peak areas were plotted against concentrations.

The linearity of the proposed method was evaluated by using calibration curve. The accuracy of an analytical method expresses the nearness between the expected value and the value found and it is determined by recovery test. In present study, successive analysis (n =3) of three concentrations (80%, 100% and120% of nominal) of working standard solution were carried out to determine the accuracy of proposed method. The precision (intra-day and inter-day)of the proposed method was expressed as %RSD amongst responses using the formula [%RSD = (Standard deviation/Mean) x 100 %]. Robustness measures the capacity of an analytical method to remain unaffected by small but deliberate variations in the parameters of the method. Robustness provides some indication of reliability of the analytical method during normal usage. The effect of the following changes in chromatographic conditions is usually determined: flow rate ±50%, solvent ratio ±10%, pH of mobile phase buffer ±0.2, temperature of the column ±10ºC and detector wavelength ±3. System suitability is commonly used to ensure the method adequacy for a particular analysis. The following parameters are verified in system suitability tests: theoretical plate count, tailing factor, resolution and reproducibility at 100% test concentration. LOD is the lowest amount of analyte in a sample that can be detected but not necessarily quantified under the stated experimental conditions. On the other hand LOQ is the lowest amount of analyte in a sample that can be

determined with acceptable accuracy and precision. These two parameters can be determined experimentally by serial dilution and visual inspection method or by following equations[17]:

$$LOD=\frac{3.3xSD}{S}$$

$$LOQ=\frac{10xSD}{S}$$

Where, SD=Standard deviation of responses and S= Slope of the calibration curve

Stressed degradation studies

In this stage, stressed degradation studies were carried out in order to degrade the sample (e.g., drug product or Active Pharmaceutical Ingredient) deliberately. These studies were carried out to evaluate the analytical method's ability for the measurement of an active ingredient and its degradation products without interference. Samples or drug product (spiked placebo) and drug substance were exposed to acid (1N HCl), base (1N NaOH), oxidizing agent(10% H_2O_2solution), reducing agent(10% Na bi-sulphite solution) and water for 24 hours at 40°C. The degraded samples were then analyzed using the method to determine if there are interferences between the active and degradation compound(s).

RESULTS AND DISCUSSION

HPLC Method Optimization and Development

Firstly, attempt was directed toward the development of an isocratic method for the simultaneous determination of these three drugs, but this attempt seemed to be apparently impossible as PARA and CAF possess close pKa value and polarity. A mobile phase with lower concentration of organic phase (~10% acetonitrile or ~30% methanol) can effectively separate PARA and CAF but takes longer period (more than 25 min) to elute IBU. On the other hand if percent of organic phase is increased to reduce the retention time of IBU (e.g. within 10 minutes) then peaks of PARA and CAF merge. We made some other efforts (such as ion pairing) to develop an isocratic method, but failed to separate PARA and CAF with good resolution while attempting to reduce retention time of all three drugs to produce a rapid analytical method. Consequently, we were directed toward the development of a gradient method and were successful. During the method development and optimization, following matters were taken into consideration:

- pKa values of PARA,CAF and IBU were 9.5,10.4 and 4.85 respectively .As a rule of thumb, pH of mobile phase buffer should be at least two unit above or below the pKa value. We could not adopt a pH value two unit above the pKa of PARA or CAF as that high pH is detremental to the silica bed of column. We could choose pH value two unit below the pKa of IBU, that is around 2.5. But at that low pH IBU remains totally undissociatedwhich is much hydrophobic

and retention time increases.Therefore finally we chose pH around 7.2 which is about two unit above the pKa of IBUand two unit below the pKa of PARA and CAF and at this pH all three drugs remain ionizedand thus their retention time were reduced to a greater extent.

- After choosing bufferpH, we tried different gradiend mode using acetonitrile and methanol. Finally we chose acetonitrile for two major reasons: firstly, acetonitrile is stronger solvent than methanol and can elute highly hydrophobic IBU early.Secondly, acetonitrile has low UV cut-off thus posing reduced baseline drift which is a common phenomenon in gradient mode.
- We set a highflow rate (1.3mL/ min) in order to reduce the re-equilibrium time.
- UV detection was set at 230 nm because above this point absorption corresponding to CAF and IBUwas reduced and below this value an increase baseline drift was shown.

Thus a rapid gradient method was developed as described in **Table 1.**Representative chromatograms were shown in **Figure 2**.

Table 1: The gradient method program for PARA, CAF and IBU

Time (min)	Buffer (%v/v)	Acetonitrile (%v/v)	Comments
0-3	90%	10%	Isocratic
3-4	90%→65%	10%→35%	Linear gradient
4-10	65%	35%	Isocratic
10-11	65%→90%	35%→10%	Linear gradient
11-15	90%	10%	Re-equilibration time

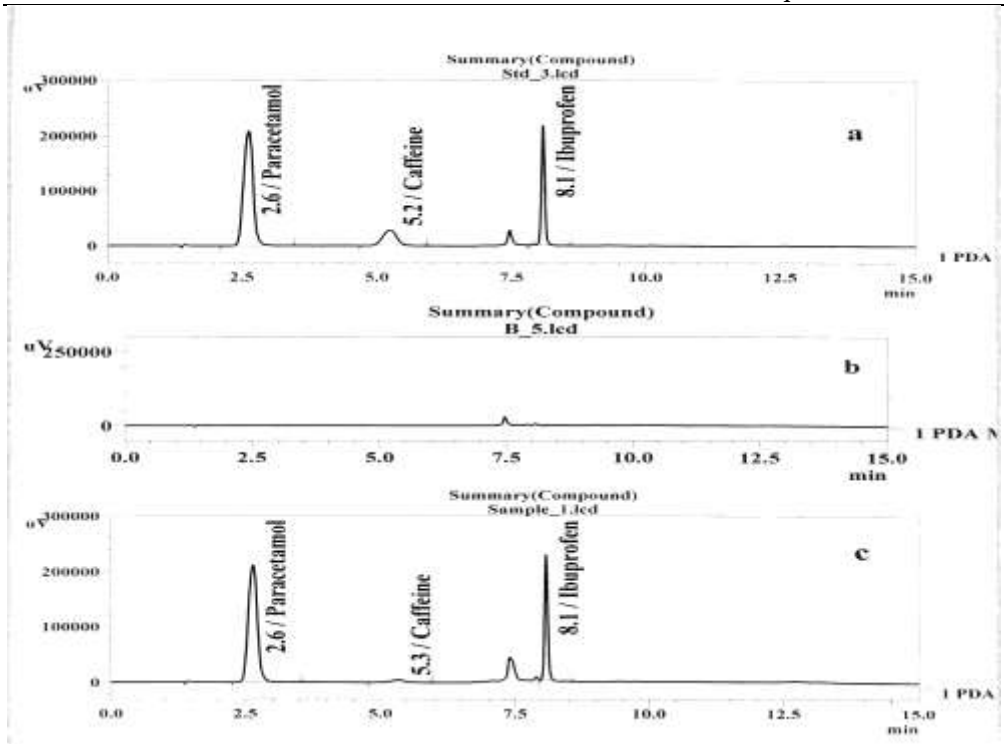

Figure 2: Chromatogram of (a) drugs in standard mixture, (b) blank and, (c) drugs in tablet sample

Solution stability

Two vials containing standard mixture, one of which was kept in room temperature and another in refrigerator. Area changes were investigated up to 2 consecutive days.% RSD of area changes in both conditions were found below the limit(1.3%) indicating the stability of drugs in diluting solution and mobile phase during analysis.

Specificity

The specificity was studied by injecting the unstressed and stressed standard solution, excipients and pharmaceutical preparation several times on several days. It was revealed that there was no interference of peak from excipients or any impurities in the region of all three drugs in the chromatogram. Chromatogram of blank and chromatograms of drugs from tablet sample were shown in Figure 2. Peak purity views presented in Figure 3 further substantiated the specificity of drugs in the chromatogram.

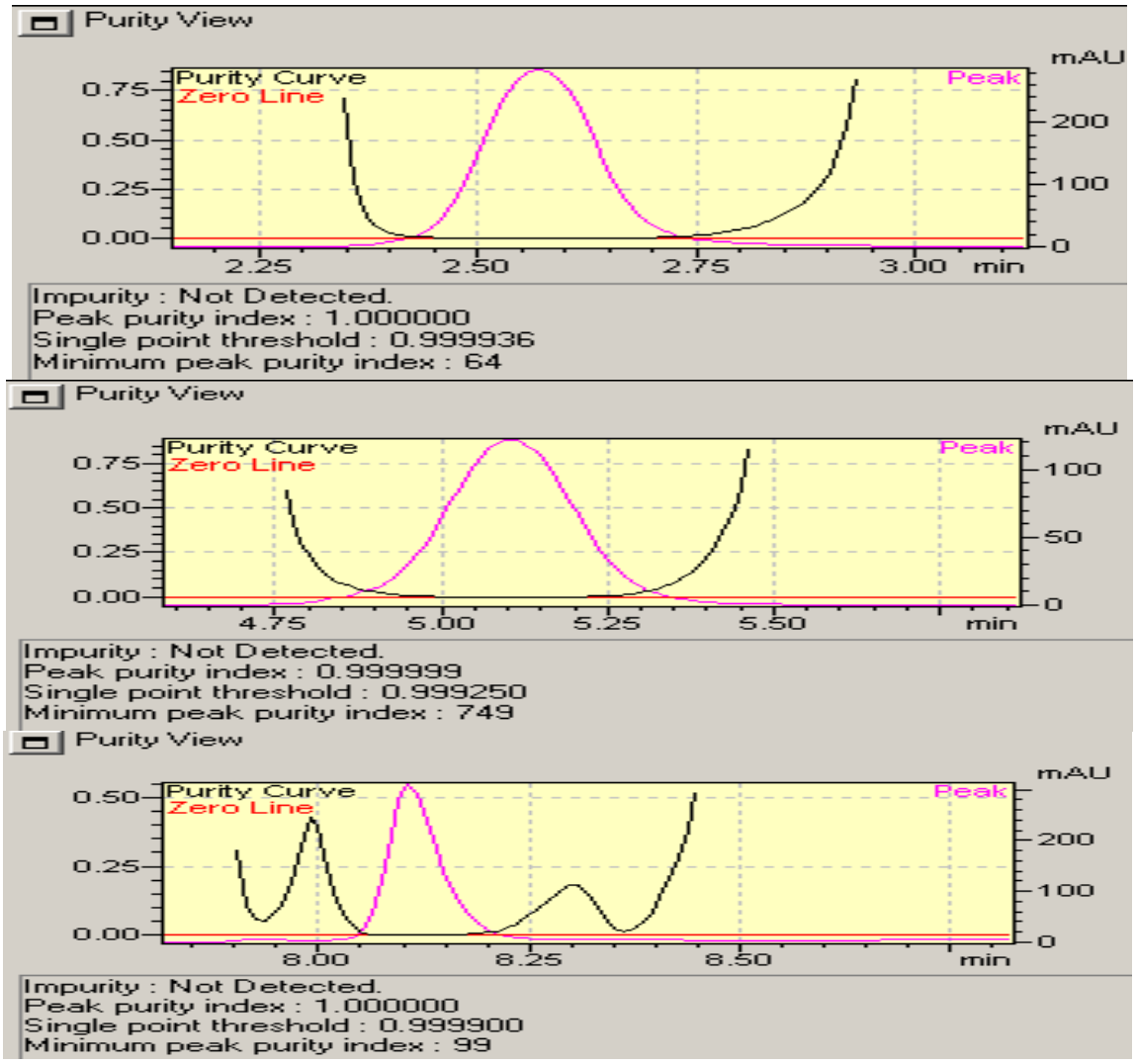

Figure 3: Peak purity index of PARA, CAF and IBU respectively.

Stressed degradation

Stressed samples were analyzed against freshly prepared standard. Degradations were measured in terms of area change between the stressed sample and freshly prepared standard. PARA was found to be degraded in significant amount by acid, base and oxidation IBU and CAF were also found moderately degraded by oxidation. One very conspicuous finding was the complete degradation of CAF in basic environment since no peak of CAF was detected. All the drugs demonstrated insignificant degradation in water hydrolysis. This result in turn substantiated our choice of mobile phase buffer pH, which is near to neutral pH at which stability of the drugs is maximum. In the chromatograms of stressed samples, the peaks of all active drugs were separated from the degraded products and ghost peaks with satisfactory resolution. Chromatograms were shown in Figure 4. Result of stressed study was summarized in Table 2.

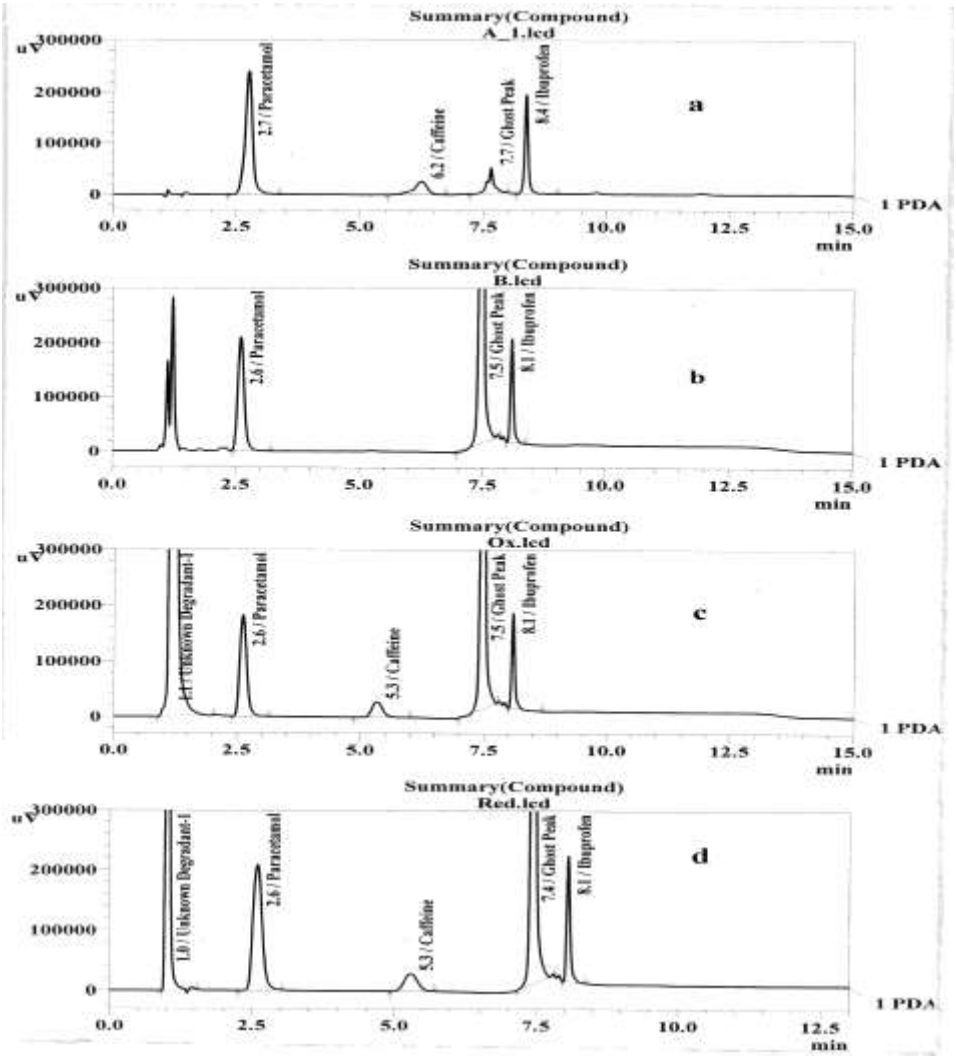

Figure 4: Stressed chromatograms of drugs and degradation products in (a) acidic phase (b) basic phase (c) oxidation medium and (d) reduction medium

Table 2: Result of stressed degradation study

Drugs	Acid Medium	Basic Medium	Oxidation Medium	Reduction Medium	Water Hydrolysis
PARA	22.8%	21.0%	26.3%	2.1%	1.1%
CAF	0%	100%	14.7%	0.8%	1.08%
IBU	3.3%	8.0%	15.3%	3.8%	0.05%

Linearity, Accuracy and Precision

The method showed good linear response of concentration with correlation coefficient (r^2) value of 0.999 for all three drugs. Accuracy of this method was calculated from the percent recovery of known concentration calculated by linear equation. The %recoveries were found above 99% for all the drugs which indicated the good accuracy of this method (Table 3). The intra-day precision (repeatability) and inter day precision (reproducibility) were measured in terms of %RSD of recovered concentrations. Maximum allowable limit for intra-day analysis is less than or equal to 2% and that for inter-day analysis (%RSD among different day, different lot of column etc) is less than or equal to 3%.The %RSD values depicted in Table3 showed that the proposed method provides acceptable intra-day and inter-day variations for these drugs.

Table 3: Results of method validation

Validation Parameters	Limit	PARA	CAF	IBU
Linear Eq.	NA	y= 51600x+8300	y=24336x-1901	y=25104x+3545
Corr. coefficient	r^2=0.998~1.0	0.999	0.999	0.999
Range	NA	25-100µg/mL	10-40 µg/mL	20-80 µg/mL
Accuracy	% recovery= 98%~102%	99.89%	100.33%	100.05%
Precision(intra-day)[b]				
Day1,Column1		0.757%	1.11%	0.789%
Day2,Column2	%RSD< 2%	0.611%	0.664%	0.630%
Day3,Column3		0.493%	0.924%	0.945%
Precision (inter day)	%RSD<3%	1.20%	2.11%	1.09%
LOD	S/N=3:1	1.99µg/mL	0.35µg/mL	0.83µg/mL
LOQ	S/N=10:1	6.03 µg/mL	1.06 µg/mL	2.50 µg/mL

[a]Mean of 6 runs, [b]Mean of three runs

System Suitability

All the system suitability parameters met the acceptance value. Table 4 summarized the results.

Table 4: System suitability parameters

Parameter	PARA	CAF	IBU	Limit
%RSD of R_t	0.734	0.571	0.117	≤2%
%RSD of area[a]	0.190	0.924	0.288	≤2%
Tailing factor[a]	1.03	1.01	1.30	≤2.0
Resolution[b]	-------	8.13	10.02	≥ 2%

Theoretical plate	1852	2778	52409	≥2000
Peak purity index	0.999	1.00	1.00	≥0.98

[a]average of six runs, [b] with respect to former peak, R_t= retention time

Robustness study

This method also showed acceptable changes in robustness study. We changed pH of mobile phase (±0.4), flow rate (±0.3mL/min) and organic composition (±10%) and observed the changes. Peak purity was retained same as that of fresh standard chromatogram in all cases and only a slight change in retention time was found. Thus this method was proved fairly robust and reliable. Result of robustness was shown in Table5.

Table 5: Summery of robustness study

Parameter Change	Actual Value	Retention time			Tailing factor			Peak purity Index		
		PARA	CAF	IBU	PARA	CAF	IBU	PARA	CAF	IBU
pH±0.4	7.6	2.7	5.5	8.12	1.13	1.2	1.02	1.00	1.00	0.99
	6.8	2.7	5.5	8.60	1.17	1.03	0.98	1.00	0.99	0.99
Flow	1.6	2.1	4.5	7.39	1.10	1.03	1.02	1.00	1.00	1.00
rate±0.3	1.0	3.4	6.9	9.50	1.14	1.00	1.14	1.00	1.00	1.00
Organic	+10%	2.5	5.0	7.82	1.01	1.00	0.98	1.00	0.99	0.99
Conc.	-10%	2.9	5.8	14.5	1.13	1.2	1.3	1.00	1.00	0.99

Application to pharmaceutical preparations

Three types of solid dosage form were formulated in laboratory to test the applicability of this method for routine analysis. Assay of the drugs was carried out by this method in the tablet formulation F1 (500 mg PARA, 400 mg IBU, 30 mg CAF), F2 (325 mg PARA, 200 mg IBU, 25 mg CAF) and capsule formulation F3 (325 mg PARA, 200 mg IBU, 30 mg CAF). Acceptable recovery value indicated the practical applicability of proposed method for routine analysis. Results were shown in Table 6.

Table 6: Assay result of pharmaceutical formulation

Formulation	Recovery [a]			%Recovery ± SD		
	PARA	CAF	IBU	PARA	CAF	IBU
F1	500.06	400.40	29.97	100.01±0.08	100.0±0.42	99.9±0.31
F2	325.16	200.30	24.87	100.05±0.13	100.15±0.5	99.48±0.06
F3	324.88	199.95	30.05	99.96 ± 0.32	99.97 ±0.31	100.16±0.21

[a]n=3

CONCLUSION

Formulation and subsequent analysis of multiple drugs has always been a challenge. Separation on HPLC becomes difficult especially when polarity and hydrophobic interaction of two drugs closely resembles each other, such as PARA and CAF. However, in present study, we were able to

develop an excellent RP-HPLC method to separate PARA, IBU and CAF simultaneously. This method was undergone careful and extensive validation process maintaining the guidelines outlined in USP, ICH and FDA and met all the requirements of a reliable analytical method. We primarily designed this method for assay analysis of those drugs in pharmaceutical preparations and in raw materials, but due to its gradient character, it can be hopefully adapted to analyze those drugs in their common dissolution medium as well.

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Competing interests

We declare no conflict of interest.

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